Influenza Viruses with Enhanced Transcriptional and Replicational Capacities

The present invention provides human influenza viruses comprising an RNA-sequence encoding a modified RNA-polymerase, a process for the preparation thereof, pharmaceutical compositions comprising said human influenza viruses and their use for gene transfer into mammalian cells, for *ex vivo* gene transfer into antigen-presenting cells, such as dendritic cells, for *in vivo* somatic gene therapy, or *in vivo* vaccination purposes. The invention also relates to other non-avian influenza viruses, including equine, porcine (swine) influenza viruses.

Background of the Invention

The RNA-dependent RNA-polymerase of the influenza virus, which is comprised of three viral polymerase (P) subunits, PB1, PB2 and PA, catalyses the synthesis of both viral mRNA (transcription) as well as complementary RNA and progeny viral RNA (replication) in infected cells (Lamb R.A., Krug R.M. Fields Virology 3: pp 1353-1445 (1996)). In the virion the enzyme is found tightly associated at each of the eight different species of viral RNAs (vRNAs) with their 5´ and 3´ ends, which in combination constitute the promoter structure, while all other parts of the vRNA molecules are covered by a large number of influenza nuclear protein (NP) molecules, one per 24 nucleotides in average (Ortega, J. et al., J. Virol. 74, 156-163 (2000)), altogether described as the viral RNP complexes. Upon infection the vRNPs are released from the virion and transferred into the nucleus of the infected cell, where viral mRNA synthesis is initiated by the promoter-associated enzyme according to the cap-snatching scheme, i.e. employing primer oligonucleotides that are derived from cellular mRNAs or hnRNAs by endonucleolytic cleavage (Krug R.M. et al., The Influenza Viruses, Plenum Press, New York, NY, pp. 1-87 (1989)). While during progression of mRNA synthesis along the vRNA

template molecule its 3´end looses contact to viral polymerase, the enzyme maintains its tight association with the 5´vRNA end throughout the entire first and all consecutive rounds of transcription. Synthesis of mRNA molecules is terminated via poly-adenylation at a 5´promoter sequence-adjacent series of 5 or 6 uridine template residues, i.e. the very 5´-terminal sequence covered by the enzyme is not transcribed into viral mRNA.

The conformation of the vRNA promoter sequence in its association with viral polymerase has been demonstrated by reverse genetic analysis to constitute a "corkscrew" structure, with exposed single-stranded tetranucleotide sequences supported by two intra-strand basepairs in both the 5´and 3´branches of the promoter sequence. In the course of that analysis also several promoter-up variants of the terminal vRNA sequence have been described, mainly through base-*pair* exchanges involving positions 3 and 8 from the 3´end, and positions 3 and 8 from the 5´end (Neumann G., Hobom G., J. Gen Virol. 76 (<pt 7):1709-17 (1995); Flick R. et al., RNA, 2(10):1046-57 (1996); Flick R., Hobom G., J. Gen Virol. 80(Pt 10):2565-72 (1999); WO96/10641). With typical increases in reporter gene or other foreign gene expression of up to 20 times the wildtype promoter yields, such influenza virus vectors range also four- to fivefold above the expression level achieved with plasmid DNAs under control of the standard cytomegalovirus early promoter (p_{CMV}).

However, there are two limitations to this effect: 1.) That gain of 20 times the wildtype promoter level in expression efficiency is true for inserts up to 1500 nucleotides in size under control of influenza promoter-up variants, while further increases in insert size up to 3000 nucleotides will steadily reduce that gain in promoter efficiency, and only low expression rates have been achieved with inserts 4000 nucleotides in length (M. Azzeh, G. Hobom, unpublished). 2.) Such increased expression rates have only been obtained as long as avian influenza virus (fowl plague viruses, FPV: H7N7) or rather viral polymerases derived from FPVs were used

together with promoter-up sequence variants; no such effect has been seen with other influenza viral strains tested : PR/8 or WSN (H1N1); Asia (H2N2) or Victoria (H3N2) (Hoffmann E., Hobom G., unpublished data).

Thus, what is needed for an application of those biotechnologically valuable increased expression rates exerted upon foreign genes in human cells or organs is a transfer of the FPV Bratislava polymerase properties in recognizing such promoter sequence variations into the respective polymerase coding sequences of other influenza viruses, able to replicate efficiently in human tissue. In addition, the use of H1N1 (WSN; PR/8) or H3N2 (Victoria or other) viral variants instead of FPV, if possible would constitute a gain in biological safety. Due to the amino acid sequence of H1 and H3 hemagglutinin-carrying viruses these become activated for infection only through cleavage by a narrow spectrum of proteases, as opposed to hemagglutinin H7 which becomes activated also through cleavage by a number of additional, ubiquitous proteolytic enzymes.

Summary of the Invention

It was found that specific modifications of the RNA sequence within the respective viruses which code for the RNA-polymerase, in particular for the PB1 subunit thereof – so as to code for a polypeptide chain having a higher similarity with FPV Bratislava RNA-polymerase – provides viruses capable of recognition of vRNA and cRNA promoter sequence variations (the so called promoter-up variants mentioned above) leading to an increase in transcription and/or replication initiation rates. The present invention thus provides

(1) a human influenza virus comprising an RNA-sequence encoding a modified RNA-polymerase which differs from the wild-type RNA-polymerase of said human influenza virus in that at least one of the amino acid residue(s) distinguishing the wild-type RNA-polymerase of said human influenza virus from FPV Bratislava RNA-polymerase has been replaced with the respective amino acid residues of FPV Bratislava RNA-polymerase ("FPV Bratislava" and "FPV Bratislava RNA-polymerase" are

hereinafter also shortly referred to as "FPV" and "FPV RNA-polymerase", respectively);

(2) in a preferred embodiment of the influenza virus defined in (1) above, the modified RNA-polymerase is capable of recognition of segments with modified vRNA promoter sequences resulting in an enhanced rate of transcription and/or replication, relative to said wild-type influenza virus RNA-polymerase;

(3) in a preferred embodiment of the influenza virus defined in (2) above, the influenza virus is suitable for high yielding expression of one or more foreign recombinant or altered viral proteins, preferably said influenza virus contains

(i) one or more segment(s) with a foreign recombinant or altered viral gene sequence in addition to the RNA segments of the normal viral genome (additional segment) or partially replacing them (hereinafter "replacing segment"), whereby the additional segment(s) and replacing segment(s) comprise the foreign or altered gene encoding the protein to be expressed in monocistronic arrangement and have modified vRNA promoter sequences as defined in (2) above; and/or

(ii) one or more bicistronic vRNA segment(s), preferably in ambisense or in tandem arrangement, whereby the bicistronic vRNA segment(s) has/have foreign gene(s) encoding the protein(s) to be expressed and being in covalent linkage with one of the authentic viral genes, preferably the neuraminidase gene, and has/have modified vRNA promoter sequences as defined in (2) above;

(4) in a preferred embodiment of the influenza virus defined in (3) above, the influenza virus has at least one segment coding for one or more foreign (or altered proper) genes in monocistronic arrangement;

(5) in a preferred embodiment of the influenza virus defined in (3) above, the influenza virus is genetically stable in the absence of any helper virus and comprises at least one viral RNA segment being an ambisense RNA molecule (hereinafter "ambisense RNA segment") and containing one of the standard viral genes in sense orientation and a foreign, recombinant

gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement;

(6) in a preferred embodiment of the influenza virus defined in (3) above, the influenza virus is genetically stable in the absence of any helper virus and comprises at least one viral RNA segment being a bicistronic RNA molecule coding for two genes in tandem arrangement (hereinafter "tandem RNA segment"), in said tandem RNA segment one of the standard viral genes being in covalent junction with a foreign, recombinant gene and said tandem RNA segment having an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region;

(7) a non-avian, non-human influenza virus, preferably an equine or a porcine influenza virus, comprising an RNA-sequence encoding a modified RNA-polymerase which differs from the wild-type RNA-polymerase of said non-avian, non-human influenza virus in that at least one of the amino acid residue(s) distinguishing the wild-type RNA-polymerase of said non-avian, non-human influenza virus from FPV Bratislava RNA-polymerase has been replaced with the corresponding amino acid residue(s) as present in FPV Bratislava RNA-polymerase, preferably said influenza virus is as defined in (2) to (6) above;

(8) a process for preparing the influenza virus as defined in (1) to (7) above, which comprises replacing the RNA-sequence encoding the wild-type RNA-polymerase of said influenza virus with an RNA-sequence encoding the modified RNA-polymerase;

(9) in a preferred embodiment of the process defined in (8) above, the process is suitable for preparing PB1-chimeric viruses as defined in (1) and (2) above as well as recombinant viruses as defined in (1) to (7) above said viruses being generated via cotransfection of up to eight cDNA plasmids containing the viral cDNAs, or chimeric (segment 2: PB1) and bicistronic recombinant (segment 6: NA/foreign gene) cDNA sequences instead, in such a way that they are transcribed *in vivo* by both RNA-

polymerase I and RNA-polymerase II and jointly give rise to progeny viruses according to the plasmid insert design;

(10) a pharmaceutical composition comprising the influenza virus as defined in (1) to (7) above;

(11) the use of the influenza virus as defined in (1) to (7) above for preparing an agent

(i) for gene transfer into cells, preferably into mammalian cells, more preferably into human cells, by viral infection;

(ii) for gene transfer into antigen-presenting cells and the use of the obtained product for *ex vivo* immunotherapy;

(iii) for *in vivo* somatic gene therapy;

(iv) for *in vivo* vaccination, including therapeutic and prophylactic vaccination;

(v) for eliciting an immune response, including the induction of T-cell response;

(vi) for treating a growing tumor or a chronic infectious disease;

(12) a method for

(i) gene transfer into cells, preferably into mammalian cells, more preferably into human cells, by viral infection;

(ii) gene transfer into antigen-presenting cells and the use of the obtained product for *ex vivo* immunotherapy;

(iii) *in vivo* somatic gene therapy;

(iv) *in vivo* vaccination, including therapeutic and prophylactic vaccination;

(v) eliciting an immune response, including the induction of a T-cell response, preferably a CD4+ T-cell response, a CD8 T-cell response or both, or the induction of an antibody response;

(vi) treating a growing tumor or a chronic infectious disease;

(vii) preparing a vaccine;

(viii) preventing and/or treating influenza;

which comprises contacting the cells (including human or mammalian cells), the antigen-presenting cells, the person or the patient in need for vaccination, influenza treatment or for somatic gene therapy, or cell cultures with the influenza virus as defined in (1) to (7) above;

(13) a method for the production of proteins or glycoproteins which comprises utilizing the influenza virus as defined in (1) to (7) above as expression vector;

(14) the use of the influenza virus as defined in (1) to (7) above for preparing agents

(i) for transfer and expression of foreign genes into cells infected by such viruses, or

(ii) for transfer and expression of RNA molecules into cells infected by such viruses, preferably the RNA molecules to be expressed are antisense sequences or double-strand sequences relative to the target cell cellular mRNA molecules, and/or the agent is suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms such as ribozyme cleavage of target RNAs;

(15) a method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with the influenza virus as defined in (1) to (7) above;

(16) the use of the influenza virus as defined in (1) to (7) above for preparing agents for immunotherapy, preferably for autologous immunotherapy;

(17) a method for an immunotherapy which comprises *ex vivo* infection of immune cells, preferably dendritic cells, with the influenza virus as defined in (1) to (7) above, and introduction of the transduced cells into the patient;

(18) a method to elicit an immune response directed against an antigen, comprising the steps of introducing the influenza virus as defined in (1) to (7) above, preferably the human influenza virus as defined in (1) to (6) above, into a cell or administering it to a mammal, wherein said influenza virus contains at least one foreign gene encoding the antigen;

(19) a vaccine for therapeutic or prophylactic purposes which is

(a) a human influenza virus vaccine comprising a human influenza virus as defined in (1) to (6) above or in (18) above, preferably said human

influenza virus encodes the antigen for a membrane protein and in addition contains the membrane protein in the viral envelope; or

(b) a non-human influenza virus vaccine, preferably an equine or porcine influenza virus vaccine, comprising a virus as defined in (7) above;

(20) transduced cells, preferably antigen-presenting cells, obtainable by the method described in (12), option (i) or (ii) above;

(21) a vaccine comprising transduced cells as defined in (20) above, preferably comprising transduced antigen-presenting cells, more preferably transduced dendritic cells, and most preferably mature dendritic cells, wherein said antigen-presenting cells are transduced *in vitro*; and

(22) a method to identify a polynucleotide sequence encoding at least one HLA-restricted epitope comprising the steps of

(a) preparing a gene bank or a cDNA bank from the cell or the microorganism to be tested;

(b) incorporating the cDNA or the DNA of the gene bank into the genome of the influenza virus as defined (1) to (7) above to yield recombinant virus particles,

(c) infecting immortalized autologous cells, which are capable of expression of HLA-class I molecules and/or HLA-class II molecules on their surface, with the recombinant virus particles obtained in step (b),

(d) expressing the proteins encoded by said cDNA or said DNA of the gene bank in the autologous cells and presenting the fragments of the proteins produced by the autologous cells or the cell surface in connection with HLA molecules;

(e) co-cultivating T-cells with the autologous cells; and

(f) stimulating the T-cells by such autologous cells which present antigens on their surface, whereby said antigens are recognized by the T-cells.

Short Description of the Figures

Figure 1: shows a comparison of variant amino acid positions in the PB1 segment of influenza A viruses, such as FPV, WSN and others, the

numbering being relative to WSN. The underlined amino acid residues representing substitutions present exclusively in WSN, while amino acid residues in bold print point out those substitutions observed only in FPV Bratislava. The complete RNA sequence of the PB1 segment of WSN is shown in SEQ ID NO: 24 (nucleotides 191 to 2461) and the corresponding polypeptide is shown in SEQ ID NO:25, while the complete sequence of FPV-PB1 is shown in SEQ ID NO:22, and the corresponding polypeptide constitutes SEQ ID NO:23.

Figure 2: Chimeric structure and determination of promoter-recognition proficiency of a first set of WSN/FPV-PB1 constructs; Sections of FPV sequence within otherwise WSN-derived PB1 are indicated in heavy lining; WSN (pPolI-WSN-PB1) and FPV (pHL3115= WF1; pHL1844) are included for comparison. Indicated in the map of PB1 are the binding sites v1 and v2 for viral RNA and c1 and c2 for cRNA in their present experimental boundaries as determined by Gonzales S. and Ortin, J. (EMBO J. 18, 3767-75 (1999)) plus the vRNA 5' and 3' terminal UV-crosslinking portions (x5' and X3'; Li, M.L. et al., EMBO J. 17, 5844-52 (1998)). Ⓟ marks the position of the polymerase active center. Major amino acid deviations are indicated showing the WSN residue on top of the FPV residue.

Figure 3: Chimeric structure and determination of promoter-recognition proficiency of a second, more detailed set of WSN/FPV constructs.

Figure 4: shows the genetic map of the FPV-Bratislava-PB1 vRNA expression plasmid used, the exact 5169 bp nucleotide sequence thereof is shown in SEQ ID NO:22 (nucleotides 191 to 2461 thereof encoding the PB1 segment of FPV Bratislava wild-type RNA-polymerase shown in SEQ ID NO:23).

Figure 5: shows the genetic map of the WSN-PB1 vRNA expression plasmid used, the exact 5169 bp nucleotide sequence is shown in SEQ ID

NO:24 (nucleotides 191 to 2461 thereof encoding the PB1 segment of WSN wild-type RNA-polymerase shown in SEQ ID NO:25).

Figure 6: shows the genetic map of PB1 chimeric plasmid pHL3102, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:26 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:27, with the following "major" modifications: L628M, V644A, and T741A and the following "minor" modifications: I576L, H584R, N633S, D636E, I645V, N654S).

Figure 7: shows the genetic map of PB1 chimeric plasmid pHL3103, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:28 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:29, with the following "major" modifications: S384P and L396I, and the "minor" modifications as pointed out in Fig. 1, positions 52 to 473).

Figure 8: shows the genetic map of PB1 chimeric plasmid pHL3130, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:30 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:31, with the following "major" modifications: S384P, L396I, L628M, V644A and T741A, and the "minor" modifications according to Fig. 1, positions 298-654).

Figure 9: shows the genetic map of PB1 chimeric plasmid pHL3131, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:32 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:33, with the following "major" modifications: S384P and L396I, and the "minor" modifications according to Fig. 1, positions 298-473).

Figure 10: shows the genetic map of PB1 chimeric plasmid pHL3203, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:34 (nucleotides

191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:35, with the following "major" modifications: L628M, V644A and T741A, and the "minor" modifications according to Fig. 1, positions 633-654).

Figure 11: shows the genetic map of PB1 chimeric plasmid pHL3204, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:36 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:37, with no "major" modifications and the following "minor" modifications: I576L and H584R).

Figure 12: shows the genetic map of PB1 chimeric plasmid pHL3246, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:38 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:39, with no "major" modifications and the following "minor" modifications: I298L and I364L).

Figure 13: shows the genetic map of PB1 chimeric plasmid pHL3247, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:40 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:41, with "major" modifications S384P and L396I, and the following "minor" modifications: D383E, H431T, N464D and L473V).

Figure 14: shows the genetic map of PB1 chimeric plasmid pHL3258, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:42 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:43, with "major" modification S384P and "minor" modification D383E).

Figure 15: shows the genetic map of PB1 chimeric plasmid pHL3259, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:44 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1

segment shown in SEQ ID NO:45, with "major" modification S384P and the following "minor" modifications: D383E, I576L and H584R).

Figure 16: shows the genetic map of PB1 chimeric plasmid pHL3268, the exact 5169 bp nucleotide sequence is shown in SEQ ID NO:46 (nucleotides 191 to 2461 thereof encoding the modified WSN RNA-polymerase PB1 segment shown in SEQ ID NO:47, with the following "major" modifications: S384P, L628M, V644A and T741A, and the following "minor" modifications: D383E, N633S, D636E, I645V, N654S).

Detailed Description of the Invention

In the present application "human influenza virus" includes all types of non-avian influenza viruses, including human, equine and porcine influenza viruses and the like, with human influenza viruses being the preferred ones. In the present application the influenza virus is also referred to as "vector", "expression vector" or "virus vector".

"Organism" embraces prokaryotic and eukaryotic systems as well as multicellular systems such as vertebrates (including mammals) and invertebrates, plants, etc. The term "cell" includes all types of cells of the "organism" defined above. A "mammal" according to the present invention includes humans and animals, "mammalian cells" include human cells and animal cells.

"Infected cells" and "infecting cells" according to the present invention also include "abortively infected cells" and "abortively infecting cells", respectively.

"Monocistronic" and "monocistronic arrangement" according to the present invention refers to a viral RNA segment, vRNA, cRNA or mRNA having one independent gene in "regular" (or "native") arrangement, while "bicistronic" according to the present invention refers to a viral RNA segment, vRNA, cRNA or mRNA that includes two independent genes in covalent junction (in a preferred embodiment of the present invention one of these genes is of viral origin, while the other one codes for a foreign, recombinant gene product).

In embodiment (1) of the invention as defined above, the influenza virus is preferably selected from influenza A including strains of type H1N1, H2N2 and H3N2, influenza B and influenza C, and more preferably is an influenza A type H1N1, including WSN/33, PR8/34 or the like, an influenza A type H2N2, including Asia/57, or the like, or an influenza A type H3N2, including Victoria/68, Aichi/68, or the like.

It is moreover preferred that the at least one distinguishing amino acid residue is located within the PB1 segment of the virus. Possible substitutions within the PB1 segment can be derived from Fig. 1. Among those, and specifically for WSN, it is preferred to replace specifically one or more of the amino acid residues at positions 384, 396, 628, 644 and 741 (based on the numbering of WSN-PB1 shown in SEQ ID NO:25) which are also designated "major modifications" or "major replacements" with the respective FPV Bratislava amino acid residues (see Fig. 1). Preferably, the influenza virus strain used is WSN-K68 carrying five distinguishing amino acids. As an alternative to or in addition to those major modifications the resulting PB1 may have one or more of the "minor" modifications as set forth in Fig. 1, namely at positions 52, 54, 105, 175, 208, 298, 364, 383, 431, 464, 473, 576, 584, 633, 636, 645 and 654. Most preferred PB1 segments are those coding for the amino acid sequences shown in SEQ ID NO: 27, 35, 43, 45 or 47.

The RNA-polymerase catalytic subunit PB1 of influenza virus WSN has been adapted by mutagenization to recognize and respond to the various nucleotide exchanges introduced in the vRNA promoter sequence that constitute promoter-up mutations in both transcription and replication. This result has been achieved through exchange of vRNA or indeed of plasmid cDNA sections within the coding sequence for polymerase subunit PB1 (segment 2) of influenza virus WSN (H1N1) using the corresponding sections derived from FPV Bratislava (H7N7) segment 2. Because of the large number of identical amino acids within both homologous segments

and the rather small fragments being switched in that construction of chimeric PB1 the genetic transfer is equivalent to an introduction of a small number of amino acid substitutions within the PB1 polypeptide chain of WSN viral RNA-polymerase, while subunits PB2 and PA remain unchanged. In principle those amino-acids required for recognition of the rather minute promoter-up variations in the template molecules should be involved in direct interactions with the respective parts of the promoter structure, in particular with nucleotides 3 and 8 from the 3´-end, which constitute one of the base-pairs in the "cork-screw" proximal promoter element. The amino acid substitutions required for that purpose are distributed over two regions that broadly are known to be involved in vRNA and cRNA binding, respectively, in two separate sections in the PB1 polypeptide chain to the left and right of the enzymatic reaction centre. No difference has been observed in virus stability, viral yields or other properties in the PB1 variants as compared to standard PB1 containing WSN virus, as long as only wildtype promoter sequences are present in all of the influenza virus RNA segments, but segments carrying promoter-up variant sequences are transcribed and replicated at elevated rates, resulting in an up to 14 times the wildtype promoted expression level.

The generation of recombinant influenza viruses was hampered for a long time by the fact that the virus has a segmented RNA genome. The development of the RNA-polymerase I technique allows the generation of recombinant viruses with additional genomic segments capable of expressing complete heterologous genes (G. Neumann et al., Virology 202, 477-479 (1994)), which was built around the *in vivo* synthesis of recombinant vRNA molecules by cellular RNA-polymerase I transcription of the respective template cDNA constructs. Modified terminal viral RNA sequences (hereinafter "promoter-up mutations" or promoter-up variants") have been designed by nucleotide substitutions (Neumann and Hobom, Mutational analysis of influenza virus promoter elements *in vivo*, J. Gen. Virol. 76, 1709-1717 (1995); WO 96/10641). The above promoter-up variants carry up to five nucleotide substitutions (in

promoter-up variant 1920; see Flick and Hobom, J. Gen. Virol. 80, 2565-2572 (1999)). When these promoter-up variants are attached to a recombinant ninth vRNA segment its increased transcription and amplification rates will not only compensate for the losses suffered spontaneously, but even cause accumulation of the foreign vRNA segment during simple viral passaging, in the absence of any selection.

As set forth in embodiment (2) above, a preferred method of the invention is where the recombinant virus contains terminal viral RNA sequences, which are active as promoter signal, have been modified by nucleotide substitution in up to 5 positions, resulting in improved transcription rates (of both the vRNA promoter and the cRNA promoter as present in the complementary sequence) as well as enhanced replication and/or expression rates relative to the wild-type sequence. Said modified terminal viral RNA sequences differ from the wild-type sequence in that in said vRNA segment the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides provided that the nucleotides introduced in positions 3 and 8 are forming a base pair (i.e., if the nucleotide position 3 is G, than that in position 8 is C; if the nucleotide in position 3 is C, than that in position 8 is G; etc.).

The 3' conserved regions of the wild-type influenza virus have the following sequences:
Influenza A: (5')-CCUGCUUUUGCU-3'
Influenza B: (5')-NN(C/U)GCUUCUGCU-3'
Influenza C: (5')-CCUGCUUCUGCU-3'.
Moreover, the 13 nucleotide conserved influenza 5'-terminal sequence may be modified by replacement of one or two nucleotides occurring in said sequence at positions 3 and 8 by other nucleotides, again provided that the introduced nucleotides are forming a base pair. The 5' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: 5'-AGUAGAAACAAGG

Influenza B: 5'-AGUAG(A/U)AACA(A/G)NN

Influenza C: 5'-AGCAGUAGCAAG(G/A):

Preferred influenza viruses of the invention are those wherein in the 3' conserved region the replacements G3A and C8U have been performed, more preferred are those where also the replacement U5C has been performed (the above mutations are annotated relative to the 3' end; such counting from the 3' end is also indicated by a line on top of the digit, e.g., G $\bar{3}$A). Another preferred influenza virus mutant comprises the 3'-terminal nucleotide sequence G3C, U5C and C8G (relative to the 3' end) resulting in the following 3' terminal nucleotide sequence (5')-CCUGGUUCUCCU-3'. Among the influenza viruses defined hereinbefore those having a 3'-terminal nucleotide sequence of (5')-CCUGUUUCUACU-3' are most preferred. In case of an influenza A virus the segment may further have the modifications U3A and A8U in its 5' terminal sequence, in case of influenza C it may have the modifications C3U and G8A in its 5' terminal sequence. The most preferred influenza viruses of the present invention comprise the following general structures:

Influenza A (mutant pHL1102):

5'-AGUAGAAACAAGGNNNU$_{5-6}$..(860-2310 ntds)..N'N'N'CCUGUUUUUACU-3'

Influenza A (mutant pHL1104):

5'-AGUAGAAACAAGGNNNU$_{5-6}$..(860-2310 ntds)..N'N'N'CCUGUUUCUACU-3'

Influenza A (mutant pHL1920):

5'-AGAAGAAUCAAGGNNNU$_{5-6}$..(860-2310 ntds)..N'N'N'CCUGUUUCUACU-3'

Influenza A (mutant pHL1948):

5'-AGUAGAAACAAGGNNNU$_{5-6}$..(860-2310 ntds)..N'N'N'CCUGGUUCUCCU-3'

Influenza B:

5'-AGUAG(A/U)AACA(A/G)NNNNNU$_{5-6}$..(860-2310 ntds)..N'N'N'N'N'(C/U)GUUUCUACU-3'

Influenza C:

5'-AGUAGUAACAAG(G/A)GU$_{5-6}$..(860-2310 ntds)..CCCCUGUUUCUACU-3'

In the above structures the variables are defined as follows:

(1) Underlined letters show the required mutations relative to the wild-type sequence for preparing a promoter mutant with enhanced properties;
(2) enlarged A in position 10 in the 5'-part of the sequence: unpaired A residue, bulge-forming;
(3) (A/G) in one position: different isolates or single segments with alternative sequence at the respective position, which are functionally interchangeable;
(4) N and N': positions undefined, but base-paired relative to each other because of complementarity between the 5' and 3' termini, different among the 8 segments, but constant for each segment throughout all viral isolates;
(5) (860-2310 ntds): the lengths of the authentic viral RNA segments, in case of segments with foreign genes increased up to 4,000 nucleotides.

Introduction of promoter-up recognition properties into standard human viruses of types H1N1 (WSN, PR/8), H2N2 (Asia), H3N2 (Victoria, Aichi, etc.) or other through PB1 mutagenization solves the problem of constructing influenza virus vectors other than FPV-derived bearing the property of expressing foreign proteins at very high rates that are suitable for expression of foreign genes in human cells or tissues including somatic gene therapy and therapeutic or prophylactic immunization.

The influenza A virus genome consists of eight segments of negative-strand viral RNA, i.e. their polypeptide coding frames are present in those vRNAs only in antisense orientation. They range in size from 890 nucleotides (segment 8) to 2341 nucleotides (segments 1 and 2). Among these the three largest segments code for three polypeptide chains that together constitute the viral RNA-polymerase: PB1, PB2 and PA, jointly comparable with RNA-polymerases as present in non-segmented negative-strand RNA viruses, which often are encoded in around 5000 nucleotides of vRNA.
Out of the three polymerase subunits that stay attached to each other in constituting the viral enzyme as present in the RNP complexes both in the

virion and in the nucleus of the infected cell, the PB1 subunit can be regarded as the central catalytic subunit, since it carries all the enzymatic functions known to date: NTP binding, RNA chain elongation, and endonucleolytic cleavage (of the cellular RNA that thereafter is used as a primer for mRNA synthesis). In addition, PB1 also includes template binding sites for both the vRNA 5´ and 3´-terminal sequences as well as the cRNA 5´ and 3´-terminal regions (Li M.L. et al., EMBO J. Oct 1; 17(19):5844-52 (1998); Gonzales S., Ortin J., EMBO J. Jul 1;18(13):3767-75 (1999)), i.e. for the various promoter elements, and finally is known to have attachment regions for both PB2 (at its C-terminus) and PA (at its N-terminus), which in turn are not directly attached to each other (Toyoda T. et al., J. Gen. Virol. Sep;77(Pt 9):2149-57 (1996); Gonzales S. et al., Nucleic Acids Res., Nov. 15;24(22):4456-63 (1996)). Subunit PB2 is known to specifically bind to the 5´-cap structure of cellular mRNA and hnRNA molecules, and thereby initiate the cap-snatching mode of viral mRNA transcription (Ulmanen I. et al., Proc. Natl. Acad. Sci. USA Dec.; 78(12):7355-9 (1981); Shi L. et al., Virus Res. Jun.;42(1-2):1-9 (1996)). Subunit PA which is known to act as a phosphoprotein appears to have a role in cRNA and vRNA synthesis, i.e. in replication, and possibly also in the corresponding switch from mRNA to cRNA synthesis (Mahy B.W.J., Genetics of Influenza Viruses, Springer Verlag, Wien, pp. 192-253 (1983); Sanz-Esquerro J.J. et al., J. Gen Virol. Mar;79(Pt 3):471-8 (1998)). Somewhat aberrantly PA if expressed in the absence of PB1 and PB2 appears to lead to proteolytic degradation of co-expressed proteins (Sanz-Esquerro J.J. et al., J. Gen Virol. Mar;79(Pt 3):471-8 (1998)).

Nuclear translocation signals have been determined within all three polypeptides, in accordance with the nuclear localization of viral polymerase in infected cells (Nieto A. et al., J. Gen. Virol. Jan;75(Pt 1):29-36 (1994)).

Interaction of viral polymerase with the 5´ and 3´ ends of vRNA or cRNA has been detected in one set of experiments through binding of ^{32}P-5´-oligonucleotides, 16-18 residues in size, which carried a single *thio*-uridine in 5´ position 15 or in 3´ position 10, respectively, followed by UV cross-linking and determination of ^{32}P-carrying peptides. In this way the 5´ vRNA sequence was observed to bind primarily to the PB1 polypeptide chain at the region centred around arginine residues 571 and 572, while the 3´vRNA model sequence attaches to the region PB1: 249-256, which includes two phenylalanine residues at positions 251 and 254 (Li M.L. et al., EMBO J. Oct 1; 17(19):5844-52 (1998)). In addition to primary vRNA binding sites as determined by UV cross-linking, in another approach secondary binding regions have also been observed for vRNA in the N-terminal region (1-139) and in the C-terminal region (493-757) using PB1 deletion variants. And while one of the cRNA binding sites determined in the same way overlaps in the N-terminal region (1-139) with vRNA binding, a second cRNA binding region is located in the central section (267-493) rather than in the C-terminal region (493-757) as detected for vRNA binding (Gonzales S., Ortin J., EMBO J. Jul 1;18(13):3767-75 (1999)).

A sequence comparison between the PB1 polypeptide chains of FPV Bratislava (H7N7) and WSN (H1N1), proficient and deficient in recognizing the promoter-up variant sequences, revealed a divergence in 22 out of 757 amino acid positions. Upon extending that comparison to include PB1 sequences also from other influenza viruses such as PR/8 (H1N1), Asia (H2N2) and Victoria (H3N2) that number of divergent positions could tentatively be reduced by 11 amino acids, which are present only in WSN and not in any other PB1 sequence in a collection of over 150 viruses representing a large variety of influenza strains. Whereas the same residue is invariably present in the respective positions both in FPV Bratislava leading to proficiency and throughout all (or most, positions 584 and 741) other isolates leading to deficiency in recognizing the promoter sequence variants. Among the remaining 11 divergent positions seven of

the FPV specific residues appear in several, but not in all other viral isolates, while four amino acid residues are specific for FPV Bratislava, and do not appear anywhere else: S384P, L396I, L628M, V644A. While the divergent positions in this category are most attractive in the search for specific properties of FPV, at this stage it cannot be ruled out that others among the altogether nine inconsistently variable amino acid residues may at least assist structurally or functionally in recognition of the variant promoter structures, in particular likely for D383E because of its adjacent position relative to S384P. The apparently most important five divergent positions cluster in two groups, in the central region (383-396) not very far from the centre of polymerization activity, and in a near C-terminal region (628-741), i.e. within the brackets of the C-terminal vRNA binding region (see Fig. 1).

In a first step of analysis we have created WSN-FPV reassortant viruses carrying either a single one of the FPV subunit vRNAs in an otherwise all WSN background, or including the three FPV polymerase subunit vRNAs simultaneously. This was achieved via direct generation of influenza viruses from a set of cloned cDNAs (Neumann G. et al., Proc. Natl. Acad. Sci. USA, Aug. 3;96(16):9345-50 (1999)) designed to be transcribed *in vivo* by cellular RNA-polymerase I into eight individual viral RNA molecules (Neumann G. & Hobom G., J. Gen. Virol. Jul;76 (Pt 7):1709-17 (1995)), which were co-transfected together with four expression plasmids for early influenza virus proteins (PB1, PB2, PA, NP; cloned in sense orientation into vector plasmid pcDNA3 (Invitrogen)). All four viral reassortants turned out to be viable even if not yielding a full titer as compared to parental WSN or FPV. They were used to determine which of the three FPV polymerase subunits was responsible for recognition of the promoter-up variant sequences and caused increased expression rates of reporter genes controlled by them. Both reassortant viruses carrying either all three polymerase subunits originating from FPV, or carrying only the PB1 subunit derived from that avian influenza virus showed increased chloramphenicol acetyltransferase activity in the transfected cells as well

as during consecutive steps of viral propagation with promoter-up variant 1104-CAT vRNAs, while the two reassortants containing either FPV-PB2 or FPV-PA in an otherwise WSN background of vRNA segments did not. From these data we conclude that it is indeed the FPV-PB1 subunit, already known from the above to interact with the vRNA and cRNA terminal sequences, i.e. the promoter structures, which is also recognizing the basepair and nucleotide substitutions present in the promoter-up variants. Another result derived from these initial experiments is the observed potential for free exchange of viral polymerase P-subunits between FPV and WSN viruses without major reduction in activity rates due to incompatibility.

Starting out from the FPV/WSN sequence comparison of PB1 and the present knowledge about the location of its functional domains a first round of chimeric PB1 clones was designed, which carried sections of both FPV-PB1 and WSN-PB1 at approximately one third and two thirds of either polypeptide chain, see Figure 2. The results confirm that the N-terminal section even though it is known to include one of the binding sites for the vRNA and the cRNA terminal sequences is not involved in promoter variant recognition, as was suggested already by the small number of amino acid exchanges, which also might be regarded to be conservative, and because all of them show up (individually) in the majority of non-proficient viruses other than WSN: R52K, R54K, T105N, N175D and R208K. Instead, the chimeric PB1 protein carrying the C-terminal FPV section (492-757) attached to the N-terminal part of the WSN polypeptide (in pHL3102), and the PB1 chimera pHL3131 carrying a central FPV region (241-492) surrounded on either side by WSN sequence resulted in high or moderately high recognition of variant promoter sequences, in accordance with being divergent by 8 or 9 amino acid exchanges, respectively. In the extended comparison of proficient versus deficient viral PB1 sequences in either case 2 substitutions thereof might be regarded to be "major" exchanges, i.e. being present only in FPV Bratislava. Because of the experimental data described below substitution T741A may also be

regarded to be in the "major" (or main assisting) category, even though the alanine residue in this position is also present in several other, non-proficient viruses.

In a second round of PB1 chimera constructions the previously used central and C-terminal FPV sections of 34 % the entire length in both pHL3131 and pHL3102 have been divided further into halves, i.e. with regard to the number of amino acid divergencies remaining in pHL3131 and pHL3102 relative to full-size WSN-PB1. In this way pHL3204 constitutes the FPV:492-599 hybrid PB1 clone, pHL3203 the FPV: 599-757 containing polypeptide chain, pHL3256 carries an FPV section extending from position 241 to position 374, and pHL3257 contains FPV sequence from position 374 to 492, see Figure 2. In addition, a selected small section of FPV-PB1 sequence (374-394) covering a most tightly clustered group of two amino acid exchanges relative to WSN-PB1: D383E, and S384P has also been inserted into WSN-PB1 both on its own: pHL3258, and in combination with a second section of FPV-PB1: 492-599 (pHL3259) or 599-757 (pHL3268), i.e. the same regions as present individually in pHL3204 or pHL3203. As documented in Figure 3, it is the latter combination of two short, separate sections of FPV carrying in one section one "major" and one "minor" substitutions, and in the other three plus four amino acids in these categories exchanged, which gave the best results in recognition of promoter-up variant 1104, with rates above each of the individual constituents, pHL3203 or pHL3258, and at 70% the level of FPV polymerase itself (see pHL1844).

The difference remaining may be due to a negative effect caused by one or more of the amino acid substitutions present in pHL3268 resulting from sterical interactions with other parts of the WSN-PB1 molecule, while that respective amino acid residue may or may not be involved directly in promoter sequence recognition, too. In the latter case the promoter activity might be further increased upon determination and elimination of that disturbing residue among the few amino acid substitutions remaining

at present. In the other case, i.e. with both effects caused simultaneously at least in part by the same residue, an improvement over the present level would not be possible. In any case the expression level for foreign proteins achieved so far for a WSN influenza virus carrying a set of only five amino acid substitutions (plus four most likely irrelevant changes) in its PB1 sequence, which in response to the standard pHL1104 mutant results in an expression rate 14 times above the wildtype promoter level (instead of 20 times for FPV), appears to be sufficient for its use in H1N1 influenza virus expression vectors.

The WSN-K68 virus carrying five constitutive amino acid substitutions in its PB1 polymerase subunit, which are modelled according to the FPV Bratislava sequence is a plaque-forming, stable virus strain indistinguishable from influenza virus WSN in its cell specificity and virus yields as long as it consists only of influenza vRNA segments carrying wildtype promoter sequences. In the presence of an (additional) influenza vRNA segment carrying a promoter-up terminal sequence the corresponding viral mRNA will be synthesized at high rates and that vRNA will be amplified disproportionately causing production of defective particles due to over-abundance of that single (foreign) viral segment over all the others. As described earlier (WO 00/53789 and EP 00115626.4) this can be brought back into a balanced, stable situation via construction of bicistronic segments, carrying the foreign gene in covalent junction with one of the viral genes, either according to the ambisense or to the tandem design. In addition, a replicational balance has to be achieved between that bicistronic segment and the set of seven regular segments through variation of the overall length of the bicistronic segment and the variant promoter sequence attached to it. With regard to transcription and consequent protein expression the lower-level yield of the viral gene product has to be brought into approximate balance with other viral gene products, while the higher yielding foreign gene product expression is maintained in imbalance with regard to the viral genes.

Alternatively, influenza virus strain WSN-K68 may be used directly as a helper virus for production of unstable recombinant viral progeny, with inherent suicide properties equivalent to attenuation. A disadvantage of that scheme is the presence of progeny helper viruses besides recombinant viruses in the supernatant of plasmid DNA transfected and helpervirus infected cells.

Construction of influenza virus strain WSN-K68 (H1N1) and expectedly of similar K68 variants of H2N2 or H3N2 viruses solves the problem of biological safety; it helps to avoid the use of H7-type viral hemagglutinin-containing viral vectors and their sensitivity to ubiquitous proteases.

The respective "major" amino acid positions in the PB1 subunit of the various parental H2N2 and H3N2 viruses mentioned earlier are largely identical to WSN, and at critical positions different from the unique sequence of FPV Bratislava (see Fig. 1), and therefore, these other viral strains are expected to become similarly converted from enhanced transcription-deficient into enhanced transcription-proficient viruses by that same procedure.

The location of the two groups of apparently crucial amino acid substitutions within the PB1 sequence overlaps in one group: L628M, V644A and T741A with one of the known binding regions for the vRNA promoter sequence, and also is in the neighbourhood of the 5´vRNA cross-link site at R571/R572. The other group of exchanges in WSN-K68: D383E, S385P, and I396L is located within the region of primary (S445/D446/D447) and in particular secondary consensus sequence elements predicted to be involved in nucleotide polymerization enzymatic activity (Poch O. et al, EMBO J. Dec. 1;8(12):3867-74 (1990); Biswas S.K., Nayak D.P., J. Virol. Mar;68(3):1819-25 (1994)). Whereas only cRNA and not vRNA terminal sequence binding has been observed in that region (Gonzales S., Ortin J., EMBO J. Jul 1;18(13):3767-75 (1999)), the

enzymatic reaction centre would have to be expected to get into close contact with both of its substrates, cRNA and vRNA.

No amino acid exchanges and hence no influence on PB1 or on viral polymerase promoter recognition properties is observed here for the N-terminal region of the PB1 polypeptide chain, which is known to interact with both the vRNA and cRNA promoter (1-143), and the same is true for the region of the 3´vRNA cross-link site (249-256). That result may be regarded to be disappointing, since the major effect on promoter-up variation originates from base-pair exchanges at positions 3 and 8 from the 3´-end. While it is obvious from the various results obtained previously with different methods and also including our own data, that widely separated parts of the PB1 polypeptide chain do interact, simultaneously or consecutively, with individual structural elements of the template molecules, and more specifically with both parts of the two RNA promoter structures, the 3D structure(s) of the entire enzyme or its PB1 subunit are not yet known.

The binding studies of Gonzales S., Ortin J., EMBO J. Jul 1;18(13):3767-75 (1999) have been done *in vitro* using large deletion variants of the PB1 polypeptide chain, which might cause severe structural deformations in the protein fragments remaining, and hence yield artifactual results. The cross-linking studies by Li M.L. et al., EMBO J. Oct. 1;17(19):5844-52 (1998) used rather short 5´ *or* 3´ oligonucleotides with a single thio-uridine residue in 5´ position 15 or in 3´ position 10. Both positions are located in the distal promoter element which is known to be double-stranded, while only single-stranded oligonucleotides have been used in these experiments. Certainly for the distal promoter element with its double-stranded RNA structure, but most likely also for the proximal promoter element, simultaneous binding of the 5´ and 3´ terminal sequence sections would have been more appropriate and might have given different results. Also, in a consecutive binding reaction of first the 5´ vRNA terminal sequence, and thereafter the 3´ vRNA end a major

conformational shift of the entire molecule has been observed (Klumpp et al., EMBO J; 16:1248-1257 (1997)). Finally, it is known that the proximal and the distal promoter elements are independent conformational units in the vRNA promoter structure, and it is the proximal element that has to be recognized in detail by polymerase, since it is the one that carries the promoter-up mutations (Flick R. et al., RNA, Oct;2(10):1046-57 (1996)). Therefore, the UV cross-linking results obtained for two nucleotide positions only in the distal element might be misleading here also for that reason. On the other hand, our *in vivo* determination of a set of amino acid positions involved in enhanced transcription proficiency versus deficiency, i.e. in recognition of single nucleotide and/or base-pair exchanges within the vRNA promoter structure (and located in full-size functional polymerase molecules) is certainly a much more gentle and more reliable way of determining polymerase functional elements than any of the *in vitro* methods used so far. The PB1 amino acids involved in recognizing that 3'-3:8 basepair and sensing a single base-pair substitution at that location, are likely to be either in direct contact with those nucleotides or at most might be located in the subsequent chain of domain interactions within the protein leading to conformational changes and the observed response, i.e. enhanced transcription initiation rates.

According to embodiment (3) defined above and the explanations given hereinbefore, the influenza virus of the invention is suitable for high yield expression of one or more foreign or altered proteins. The foreign recombinant or altered viral gene may be present within an additional RNA segment or in a replacing segment, which comprise the foreign or altered gene encoding the protein to be expressed in monocistronic arrangement and have a modified vRNA promoter sequence as defined above (embodiment (4)), and/or within a bicistronic vRNA segment, preferably in ambisense or in tandem arrangement (embodiments (5) and (6), respectively), which includes the foreign gene encoding the protein to be expressed and has a modified vRNA promoter sequence as defined above.

Concerning embodiment (4), which relates to the expression of foreign glycoprotein genes and incorporation of those glycoproteins in the viral envelopes, it is referred to DE 197 09 512 (the disclosure thereof is herewith incorporated by reference). According to said embodiment the genes of different foreign glycoproteins of general type I, i.e., with a hydrophobic membrane anchoring sequence close to the C terminus, can be used for corresponding cDNA constructions, as designed for the expression of other genes, so that corresponding artificial vRNA molecules can be formed, i.e., in minus strand orientation. The foreign genes are inserted instead of the coding sequence of an influenza gene, flanked by authentic or slightly modified non-coding regions, as present in the influenza vRNA molecule. In addition to its own signal peptide sequence or the one borrowed from hemagglutinin (HA), the recombinant glycoprotein sequence then comprises its complete own, i.e., HA-foreign, ectodomain, followed by either its own transmembrane domain or, more frequently, that of hemagglutinin including its C-terminal "cytoplasmic" tail sequence (for HA: 26 + 11 amino acids). The same principle of construction applies to non-glycoproteins which may be converted into artificial surface proteins by being connected with the two flanking signal peptide and membrane anchor elements derived from viral hemagglutinin.

Concerning embodiment (5) it is referred to WO 00/53789 (the disclosure thereof is herewith incorporated by reference). In the influenza virus of said embodiment preferably at least one of the regular viral RNA segments is replaced by an ambisense RNA segment which contains one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation or vice versa in overall convergent arrangement. It is moreover preferred that in the ambisense RNA molecule said foreign recombinant gene is covalently bound to one of the viral genes while the original vRNA segment coding for the same gene is deleted from the recombinant virus by a specific ribozyme cleavage, or left out from the set of RNA-polymerase I viral cDNA clones and substituted by the

corresponding ambisense RNA expressing cDNA clone in the process of direct generation of recombinant influenza viruses (see below).

The foreign gene(s) in ambisense covalent junction with the viral gene(s) preferably code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus, such as lymphokines or extracellular enzymes, or code for glycoproteins that are incorporated into the viral envelope as well as the plasma membrane of the infected cell. In another preferred embodiment the foreign gene(s) in ambisense covalent junction with the viral gene(s) code for proteins or artificial polypeptides designed to support an efficient HLA-restricted presentation of inherent epitopes at the surface of infected cells, for stimulation of B cell and/or T cell response. Such proteins or artificial polypeptides constitute for instance a tumor antigen or an artificial oligomeric series of T cell epitopes. Finally, the foreign genetic insert(s) may be suitable for transfer and expression of RNA molecules, including antisense RNAs and ribozymes within the infected cells. Such recombinant influenza viruses are suitable for sequence specific gene silencing, for example by RNA antisense or ribozyme RNA interference mechanisms.

A preferred virus of embodiment (5) of the invention is where in the regular viral RNA segments one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged into foreign glycoprotein(s), or preferably into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

Concerning embodiment (6) of the invention it is referred to EP 00115626.4 (the disclosure thereof is herewith incorporated by reference). In the tandem RNA segment of said embodiment, one of the standard viral genes is preferably in covalent junction with a foreign,

recombinant gene and said tandem RNA segment has an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region. "Proximal" and "proximal position" according to the present invention refers to the 5' position of one of the genes in the bicistronic viral mRNA, i.e., ahead (upstream) of the second gene in "distal position".

Expression of both gene products in the tandem constructions is made possible by way of an upstream splice donor and a downstream splice acceptor signal surrounding the proximal coding region of such a quality that splicing does occur in part of the mRNA molecules only, i.e., both mRNAs spliced and unspliced are present in the infected cell. For compensation with regard to the vRNA length the bicistronic segment is connected to a promoter variant of enhanced replication and transcription rates as defined hereinbefore.

The splice donor and the splice acceptor signals are selected from authentic sequences as present in influenza segments 7 and 8 or other partially effective splice reaction substrates, preferably those of influenza virus WSN segment 7, i.e., 5'-AG$^{\downarrow}$GTACGTTC-3' (donor) and 5'-GCTGAAAAATGATCTTCTTGAAAATTGCAG$^{\downarrow}$GC-3' (acceptor).

In a preferred influenza virus according to embodiment (6) at least one of the regular viral RNA segments is replaced by a tandem RNA segment which contains one of the standard viral genes in distal position, and a foreign, recombinant gene in proximal position, both in anti-sense orientation, or vice-versa. It is moreover preferred that the same viral gene as present in the bicistronic RNA segment is deleted from the recombinant virus by specific ribozyme cleavage or is left out from the set of RNA-polymerase I cDNA clones and substituted by the corresponding tandem bicistronic RNA expressing cDNA clone in the direct generation of recombinant influenza viruses from plasmid DNAs (see below).

The foreign gene(s) in tandem covalent junction with the viral gene(s) preferably code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus, such as lymphokines or extracellular enzymes, or code for glycoproteins that are incorporated into the viral envelope as well as the plasma membrane of the (abortively) infected cell. In another preferred embodiment the foreign gene(s) in tandem covalent junction with the viral gene(s) code for proteins or artificial polypeptides designed to support an efficient HLA-restricted presentation of inherent epitopes at the surface of infected cells, for stimulation of B cell and/or T cell responses. Such proteins or artificial polypeptides constitute for instance a tumor antigen or an artificial oligomeric series of T cell epitopes that have been identified within a polypeptide chain. Finally, the foreign genetic insert(s) may be suitable for expression of RNA molecules, including antisense RNAs and ribozymes, within the infected cells. Such recombinant influenza viruses are suitable for sequence specific gene silencing, for example by RNA antisense or ribozyme interference mechanisms.

A preferred recombinant virus of embodiment (6) of the invention is where in the regular viral RNA segments one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged into foreign glycoproteins, or preferably into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

According to embodiments (5) and (6) the invention provides
- a stable recombinant influenza virus containing (up to) seven regular vRNA segments plus one (or more) additional bicistronic segment(s) coding for a foreign gene in covalent conjunction with one of the influenza genes, in ambisense or in tandem arrangement, and

- a method for the construction of stable recombinant influenza viruses through (a) bicistronic vRNA segment(s) in ambisense or in tandem arrangement, that is also applicable as a method for attenuation and for prevention of reassortment between co-infecting influenza viruses.

In a particular application of embodiment (6) the tandem bicistronic mRNA codes for one of the viral genes, such as hemagglutinin, in conjunction with (all or) part of the viral neuraminidase coding sequence or the viral NS1 coding sequence, in inverted (antisense) orientation, while the authentic neuraminidase vRNA segment or NS1 coding sequence is otherwise missing entirely in these recombinant viruses. In another variation of these constructs an anti-neuraminidase or anti-NS1 ribozyme sequence is also provided together with the (partial) neuraminidase or NS1 antisense sequence, in the proximal or in the distal position of these bicistronic recombinant segments. Recombinant viruses of this character are propagated in culture media with addition of exogenous neuraminidase or in tissue culture cells with inactivated interferon genes, e.g., Vero cells.

The absence of a functional neuraminidase gene serves as a strong attenuation mechanism resulting in single-step infections of such recombinant viruses only. While a functional neuraminidase gene could be provided in case of another (wildtype) influenza virus superinfecting the same cell, expression of that gene is very much reduced through antisense RNA interaction and/or destruction of the corresponding vRNA through ribozyme cleavage, designed to interfere with production of infectious progeny even from co-infected cells; as a barrier against reassortment in double infected cells. The same argument applies for the NS1-deleted viruses which in addition carry an anti-NS1 vRNA antisense or ribozyme sequence, as a second feature besides carrying a foreign recombinant gene in ambisense or tandem bicistronic design.

Recombinant viral RNAs coding simultaneously for two genes in tandem organization within a construct, in which one of the viral genes is

connected in covalent junction with a foreign coding sequence, are constructed via E. coli plasmid vector DNAs designed for an *in vivo* transcription of minus-strand vRNAs by cellular RNA-polymerase I. In these constructs the gene in plus-strand proximal (upstream) position is surrounded by splice signals of limited activity such that both mRNAs, spliced and unspliced are present in the infected cell. Either the foreign gene or the viral gene may be in that upstream position. In the majority of applications the higher rate of expression will be reserved for the foreign coding sequence, while the lower expression rate of the viral gene is adapted to be approximately in balance with expression of the other viral genes encoded by the regular viral segments.

To achieve such a balanced rate of expression, the splice signals and the promoter have to be chosen properly (Flick and Hobom, Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation, J. Gen. Virol. 80, 2565-2572 (1999)). At an increased overall transcription rate, the resulting mRNAs shall be spliced inefficiently if the viral gene is in the distal (downstream) position. Vice-versa, if the foreign gene is in the distal position, splicing to obtain the foreign mRNA shall be achieved efficiently. Both designs serve to reach an over-expression of the foreign gene relative to its companion viral gene, of which the expression shall be in balance with the expression of the other viral genes. Further, the promoter variant attached to the bicistronic segment has the function to compensate for the increased gene length by way of an increased replication rate.

The influenza vRNA segments preferably used for construction of bicistronic segments include the neuraminidase (No. 6), hemagglutinin (No. 4) and NS segment (No. 8). In the NS segment the foreign gene may also substitute for the NS1 gene, leaving the viral NS2 gene in its place. These recombinant viruses can, as an example, be made by the following procedure: A recombinant virus population can be selected by repeated ribozyme-mediated cleavage of a helper-virus segment carrying inserted

ribozyme cleavage sites in flanking positions of the same viral gene in the monocistronic segment as has been included in the bicistronic construct (PCT/EP00/01903). By serial viral passaging and relying on the ouptut of reporter genes in equivalently constructed bicistronic segments, a balanced mode of expression can be achieved in choosing the right set of elements: promoter variant, splice signals, plus a limited variation in segment length. The construct that gives rise to the balanced, stable expression may then be used for designing a multiple cDNA transfection procedure of helper-free generation of influenza viruses according to Hoffmann et al., Proc. Natl. Acad. Sci. USA, Vol 97, 6108-6114 (Mai 2000). The resulting recombinant influenza virus, obtained via single plaque in pure helper-free state, is subjected to another series of propagation steps to finally evaluate its properties.

In a particular application this design is used for establishing a controlled mode of viral attenuation. Attenuation of influenza viruses so far has been achieved in cold-sensitive mutants (Edwards et al., J. Infect. Dis. 169, 68-76(1994)), by (partial) deletion of the NS1 gene (partial attenuation, Egorov et al., J. Virol. 72, 6437-6441 (August 1998) and Palese et al., Proc. Natl. Acad. Sci USA, 4309-4314 (April 2000)), or through deletion of the neuraminidase gene (full attenuation, Kawaoka et al., J. Virol. 74, 5206-5212 (June 2000)). The latter approach is adapted here, together with a novel technique for viral attenuation, which for the first time is also able to interfere with (chance) superinfection by wild-type viruses.

In this embodiment of the invention a second bicistronic cDNA construct is designed, which instead of carrying another foreign gene is coding for part of the viral neuraminidase gene in antisense orientation, with or without being surrounded both by splice donor and acceptor elements. In another version of that design a 2 x 50 nucleotide antisense segment complementary to a region of the neuraminidase sequence has been cloned in flanking positions relative to a ribozyme construct according to the hammerhead design and oriented against a common GUC triplett

within the neuraminidase sequence in a majority of current post-viral isolates. In a preferred design this antisense expression construct has been attached to the hemagglutinin vRNA segment, while another gene or reporter gene is encoded in a second bicistronic vRNA, in conjunction with NS2. The same design applies for an anti-NS1 virus, which itself does not carry an NS1 gene, and has a foreign recombinant gene in conjunction with neuraminidase vRNA or NS2 vRNA.

Propagation of recombinant viruses deleted for the neuraminidase (NA) gene requires an addition of external neuraminidase to the medium. In the absence of neuraminidase, infection by the NA deletion viruses is abortive: no infectious progeny is produced. Upon co-infection (3 + 3 per cell) of recombinant viruses together with wildtype viruses no progeny virus or plaque is observed, which is attributed to antisense-blocked expression and/or (partial) ribozyme destruction of the neuraminidase segment originating from the wild-type virus. According to this design, the recombinant viruses described are not only attenuated in single infections, but simultaneously interfere with wildtype virus superinfection, and therefore, no re-assortment between the two viruses will occur.

Concerning the process of embodiment (8) it is referred to the disclosure of WO 96/10641, PCT/EP00/01903 and EP 00115626 referred to above (the disclosures of which are herewith incorporated by reference) and the detailed discussion of the production method set forth above.

Concerning the embodiment (9) a final step in the generation of WSN/FPV-PB1 chimeric viruses such as influenza WSN-K68 consists in performing an eight cDNA plasmid-cotransfection into 293T cells designed according to Hoffmann et al (Hoffmann E. et al., Proc. Natl. Acad. Sci. USA 97 (11), 6108-6113 (2000)), i.e., carrying inserts cloned into vector plasmid pHW2000 or an equivalently organized plasmid vector containing flanking RNA-polymerase I as well as RNA-polymerase II transcription units, in opposite direction to each other and extending across the central

cDNA insert. The inserts used in a set of eight dual expression plasmids constructed accordingly consist of wildtype WSN cDNAs derived from the 7 segments except for segment 2 (PB1), which was constructed using PB1 chimeric plasmid pHL3268 as a starting material. Cotransfection with all eight plasmids after 72 hours resulted in virus containing supernatants, which can be used for plaque purification and further propagation on MDCK cells, and for characterization of the viral isolate by RT-PCR followed by restriction analysis or DNA sequencing.

Generation of recombinant influenza viruses followed the same outline, but in this case one of the WSN segments, preferably segment 6 (NA) has been exchanged for a bicistronic construct containing the NA gene in covalent junction with the foreign gene, in ambisense or in tandem design. In addition, the bicistronic segment carries promoter-up mutations in its flanking 5' and 3' terminal sequences. Other recombinant viruses have been created using a bicistronic segment 8 (NS2/foreign) with or without an anti-NS1 ribozyme sequence inserted in a flanking position in segment 6, and still other of the segment 8 bicistronic viruses (NS2/foreign) have their segment 6 deleted entirely, with or without an anti-NA ribozyme sequence inserted in a flanking position of segment 4(HA).

The pharmaceutical composition according to embodiment (10) above and the agent of embodiments (11), (14) and (16) above (hereinafter also referred to as "medicament") contain the recombinant influenza virus in a pharmaceutically effective amount. Besides said recombinant influenza virus, the pharmaceutical composition and the medicament may contain further pharmaceutically acceptable carrier substances well-known to a person skilled in the art, such as binders, desintegrants, diluents, buffers, preservatives, etc. The pharmaceutical compositions and medicaments are solid or liquid preparations and are suitable to be administered orally, intravenously or subcutaneously. For treatment of humans a human influenza virus according to embodiments (1) to (6) is preferably used.

The pharmaceutical composition of embodiment (10) is, among others, suitable

(i) for gene transfer into cells, preferably into mammalian cells, more preferably into human cells, by recombinant viral infection (namely via standard viral infection or employing in addition a specific attenuation mechanism);

(ii) for gene transfer into antigen-presenting cells, preferably into dendritic cells, and the use of the obtained product for *ex vivo* immunotherapy (whereby *ex vivo* therapy is therapeutic application involving the modification of antigen-presenting cells, such as dendritic cells, and wherein the modified antigen presenting cells are injected directly into the patient);

(iii) for *in vivo* somatic gene therapy;

(iv) for *in vivo* vaccination, including therapeutic and prophylactic vaccination;

(v) for eliciting an immune response, including the induction of a T-cell response;

(vi) for treating a growing tumor or a chronic infectious disease.

The pharmaceutical composition of embodiment (10) and the medicament according to embodiment (11) above is preferably suitable as a medicament against influenza and/or against other infections. The recombinant influenza virus may be present in form of inactivated preparations or may be present in form of live recombinant viruses, preferably as attenuated viruses.

Live recombinant viral vaccines, live but attenuated recombinant viral vaccines or inactivated recombinant viral vaccine can be formulated. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity is destroyed without affecting its immunogenicity. For preparation of inactivated vaccines, the recombinant virus may be grown in cell cultures or in embryonated chicken eggs,

purified, and inactivated by formaldehyde or ß-propiolactone. The resulting vaccine is usually administered intramuscularly.

Inactivated viruses may be formulated with suitable adjuvants to enhance the immunological response. Such adjuvants include, but are not limited to, mineral gels, e.g., aluminum hydroxide, surface-active substances such as pluronic polyols, lysolecithin, peptides, oil emulsions, and potentially useful human adjuvants such as BCG.

Many methods may be used to introduce the vaccine formulations above, for example the oral, intradermal, intramuscular, intraperitoneal, subcutaneous, or intranasal routes. Where a live recombinant virus vaccine is used, it is preferred to introduce the formulation via the natural route of infection for influenza virus.

The agent according to embodiments (11), (14) and (16) above is preferably suitable for prophylactic or therapeutic vaccination, or both, against influenza and other infections. For example, recombinant viruses can be made for use in vaccines against HIV, hepatitis B virus, hepatitis C virus, herpes viruses, papilloma viruses, to name but a few. In one embodiment the recombinant virus contains the genes for surface proteins of the viruses, in another the genes for non-structural or regulatory genes. The recombinant viruses may be present in form of inactivated preparations or may be present in form of live recombinant viruses, or as live, but attenuated viruses. In an attenuated virus the recombinant virus would go through a single or at most very few propagation cycle(s) and induce a sufficient level of immune response, but would not cause disease. Such viruses lack one of the essential influenza genes or contain mutations to introduce temperature sensitivity.

The agents of embodiments (11), (14) and (16) above of the invention are applicable in *ex vivo* and *in vivo* application schemes. The RNA molecule to be expressed by means of the agent of the embodiment (14) is of an

antisense sequence or double strand sequence (in ambisense bidirectional transcription) relative to a target cellular mRNA molecule. In embodiment (14) the agent is preferably suitable for sequence-specific gene silencing, preferably by RNA antisense or ribozyme interference mechanisms.

The method for the production of proteins or glycoproteins of embodiment (13) is preferably performed in tissue culture cells or in fertilized chicken eggs, in accordance with standard techniques within the general knowledge of a person skilled in the art. The proteins or glycoproteins to be expressed are those incorporated into the virus, in monocistronic or bicistronic arrangement as defined hereinbefore.

The methods according to embodiments (12), (17) and (18) of the invention include the administration of an effective amount to the mammal (i.e. the patient in need for vaccination, for influenza treatment or for somatic gene therapy) or the administration of a sufficient infective dose of the recombinant virus to the cell system (including antigen-presenting cells, cell cultures, etc.) that is used for *ex vivo* therapy or for *in vitro* investigations, whereby the amount and dose will be determined by a person skilled in the respective arts or knowledgeable of the desired treatments. For treatment of human patients the use of human influenza viruses as defined hereinbefore is preferred.

The method of embodiment (18) is disclosed in EP 00123687.6 (which is hereby incorporated by reference). In particular, said method comprises expression of one or more tumour-associated antigens (TAA) or virus-associated antigens (VAA) by dendritic cells by

(a) preparing a recombinant influenza virus containing a nucleotide sequence coding for the TAA or VAA, and

(b) infecting dendritic cells with the recombinant influenza virus obtained in step (a).

The nucleotide sequence coding for the TAA or VAA may be present in the recombinant influenza virus in one of the regular segments in bicistronic

arrangement or as an additional segment as explained in detail herein-before.

Vaccination with dendritic cells presenting tumor antigens will induce a potent primary immune response or amplify existing cytotoxic antitumor T cell responses. Therefore, tumor antigens most suitable for immunotherapy are, ideally, strictly tumor specific, or at least the immune response should have tumoral specificity. Many tumor antigens, however, are shared by tumors with normal cells and are only overexpressed in the tumor. This implies that an immune response could potentially be harmful, if an immune response to self-antigens occurs causing autoimmunity. The technology for DC vaccines shall thus result in an immune response of sufficient quality and magnitude of tumor-specific T cell responses.

Tumor-specific antigens are rare. However, a growing family of testicular antigens has been identified that are aberrantly expressed in a significant proportion of tumors of various histological types and -in addition- only in testis cells. These antigens, called cancer/testis antigens, discovered by scientists of the Ludwig Institute for Cancer Research (LICR) in Brussels and New York, and their collaborators, should ensure strict tumoral specifcity of the immune responses as the germ line cells do not express MHC-I molecules.

These antigens, for example the MAGE-, GAGE- and BAGE-families, NY-ESO-I or HOM-MEL-40 (aka SSX-2) are thus prime candidates for the DC-based antitumor vaccines, when part of a potent dendritic cell vaccine based on influenza virus-mediated gene transfer. These antigens have the required selectivity for a flu-vector based DC vaccine, can most likely be readily incorporated into the recombinant virus, and are able to induce cellular immune responses. Epitope peptides derived from MAGE-A3 have been HLA-attached ("loaded") to the dendritic cells by Schuler and coworkers and the vaccine was found to induce specific CTL in patients and *in vitro*.

Furthermore, the number of tumor antigens suitable for potent therapeutic vaccines is still limited and a search for novel tumor antigens, as well as viral antigens, seems warranted. The influenza virus vector system is suitable for antigen discovery (see above). Co-expression of said antigens with LICR antigens is an important option for widening the vaccine spectrum.

In experiments it was shown that genetically modified DC, which express tumor-associated antigens can efficiently induce anti-tumour immunity and thus have a high potential as tools in cancer therapy. The gene delivery is most efficiently achieved by viral vectors. Genes encoding a melanoma derived TAA, such as MAGE-3, or the green fluorescence protein (GFP) were introduced into a high-expression avian influenza virus vector. Monocyte-derived mature DC infected by these recombinants efficiently produced GFP or MAGE-3. More than 90 % of the infected DC can express a transduced gene. Importantly, these transduced DC retained their characteristic phenotype, their potent allogeneic T cell stimulatory capacity and were able to stimulate MAGE-3 specific $CD8^+$ cytotoxic T cells. Thus influenza virus vectors provide a highly efficient gene delivery system in order to transduce human DC with TAA, which consequently stimulate TAA specific T cells.

The agent of embodiment (17) of the invention is preferably utilized to infect, transfect or transduce patient-derived immune cells. The agent is suitable for treatment of cancer or chronic viral infections. For this purpose, patient derived immune cells, preferably dendritic cells, are *ex vivo* infected with recombinant influenza viruses expressing, e.g., tumor antigens or viral antigens. The transduced cells are then reintroduced into the patient.

The preferred method for immunotherapy of embodiment (18) of the invention is an autologous immunotherapy, wherein the cells which are *ex vivo* infected are patient-derived and the transduced cells are reintroduced

into the patient. The diseases to be treated by this method include cancer and chronic viral infections. For details regarding preparation of the treatment with dendritic cells see discussion of embodiment (12) above.

The method for inducing an immune response against an antigen according to embodiment (18) of the invention is suitable for inducing antibodies to foreign proteins including glycoproteins, following the administration of protein or glycoprotein antigens as part of a recombinant influenza virus in an authentic conformation, whereby the virus is purified by gentle procedures based on hemagglutination, and the gene is expressed at high rates in the infected cells. Suitable foreign genes encoding one of these antigens are polynucleotide sequences associated with a disease, preferably an infectious disease or tumor disease, preferably the antigen is exemplified by, but not limited to,

(i) virus-associated antigens such as the HIV antigens gp160, gp 120, rev, tat, NC, the HBV e-antigen or core antigen, the HPV E6 or E7 antigen, the herpes simplex virus glycoproteins or core proteins, other herpesvirus antigens and further viral and microbial antigens known to those skilled in the art,

(ii) tumor associated antigens, especially the so-called cancer testis-antigens exemplified by the MAGE, BAGE and GAGE family of antigens, the NY-ESO-1 antigen, the SSX antigens, exemplified by the HOM-MEL-40.

The above polynucleotide sequences

(i) are derivable from cDNA libraries isolated from tumor cells, or testis cells, or virus-infected cells, or micriobially infected cells, or cell-lines,

(ii) are fusion proteins with the hemagglutinin membrane anchor sequence, or polypeptides consisting of epitopes derived from one or more T-cell specific epitope sequences as present in viral or other pathogens, or in tumor associated antigens.

As influenza viruses have a wide host range, recombinant influenza viruses can be used to obtain strong immune responses in, and isolate antibodies from, a wide range of animals, including, but not limited to, fowl, pigs, horses, seals and mice. Further, influenza viruses adapted to the mouse can be used for the infection of mice by several routes including the intranasal route. This results in infection of the pharyngeal mucosal cells and results in an additional type of B cell response (e.g., as recognized in the ratio of IgG to IgA). Mice are of particular utility in the induction of immune responses in transgenic mice that have been engineered to express human antibodies. As gentle procedures based on hemadsorption are used to purify influenza viruses, antibodies to antigens in native conformation can be isolated from the infected mammals.

The vaccines according to embodiments (19) and (21) of the invention may contain further ingredients, e.g., those set forth with regard to the pharmaceutical composition or agents set forth above.

Concerning embodiment (22) of the invention it is referred to PCT/EP00/09217, which is herewith incorporated by reference. The method of embodiment (22) is also suitable to study gene function in antigen presenting cells.

The present invention is hereinafter described in more detail by way of the following description of the figures and examples, which are, however, not to be construed so as to limit the invention.

Detailed Description of the Tables and Figures

Table 1

Viral strains	Origin of segments					pHL1844-CAT-activity	
	PB1	PB2	PA	NP	other	293T	MDCK (1)
WSN	WSN	WSN	WSN	WSN	WSN	10	<2
WF1	FPV	WSN	WSN	WSN	WSN	21	30
WF2	WSN	FPV	WSN	WSN	WSN	6	<2
WF3	WSN	WSN	FPV	WSN	WSN	8	<2
WF4	FPV	FPV	FPV	WSN	WSN	26	35
FPV	FPV	FPV	FPV	FPV	FPV	41	**100**

Table 1. Reassorted viral strains used as helper viruses in infection of plasmid pHL1844 transfected 293T cells, followed by propagation of resulting recombinant progeny virus in MDCK cells.
Chloramphenicol acetyl-transferase enzymatic activities in 293T and MDCK (first viral passage) cell lysates, relative to standard FPV (=100).

Figure 1: Comparison of relevant PB1 amino acid positions in a selected set of influenza virus strains. The 22 amino acid positions divergent between FPV Bratislava (line 1) and WSN/33 (line 3) have been compared in the context of a large, representative group of viral isolates. Amino acid residues present only in WSN and in no other PB1 sequence have been underlined, while those four amino acid residues characterizing FPV Bratislava as approved to all other strains are given in bold print.

Figure 2: Chimeric structure and determination of promoter-recognition proficiency of a first set of WSN/FPV-PB1 constructs; Sections of FPV sequence within otherwise WSN-derived PB1 are indicated in heavy lining; WSN (pPolI-WSN-PB1) and FPV (pHL3115; pHL1844) are included for comparison. Upon generation of chimeric viruses in 293T cells stocks have been prepared on MDCK cells. 293T cells transfected by plasmid pHL1844 which carries the standard promoter-up variation as present originally in

pHL1104 have been infected by the various viral constructs as helper viruses at m.o.i. 1 (with viral strains derived from pHL3130 and pHL3115 only at m.o.i. 0.2 and 0.5, respectively). The resulting supernatants containing the viral progeny were used for viral passage onto MDCK cells. Cell lysates from both transfected and/or infected cells were used for CAT activity measurements relative to pHL1844 recombinant FPV viruses in MDCK cells (=100), known to recognize promoter-up variant pHL1844.

A selection of functional elements within the PB1 protein sequence together with the position of six "major" amino acid substitutions have been indicated in the first line of the figure. Proposed binding regions for the vRNA (v1, v2) or the cRNA (c1, c2) promoter, and known cross-linking sites for the 5´(x5) and 3´(x3) vRNA termini are marked. P refers to the active center of polymerase activity (PB1: 445-447). Amino acid exchanges are indicated by the WSN residue on top of the FPV residue, e.g. S/P refers to amino acid substitution S384P (WSN/FPV).

Figure 3: Chimeric structure and determination of promoter-recognition proficiency of a second, more detailed set of WSN/FPV constructs. For descriptions of experimental details, see Fig. 2.

Examples

Plasmid pHL1844: The CAT-gene containing expression plasmid pHL1844 has been designed for pseudo-vRNA synthesis by human RNA-polymerase I and carries the human rDNA core promoter region (-411 to –1) plus the murine rDNA terminator region (+ 566 to + 785 relative to the mature 28S rRNA 3´-end), both in flanking positions relative to the cDNA insert. The cDNA construct inserted in between consists of the 5´and 3´noncoding sequences as present in FPV vRNA segment 5, with the NP coding sequence itself being exchanged for the coding sequence of chloramphenicol acetyltransferase (CAT) of bacterial origin, in antisense orientation relative to RNA-polymerase I transcription. pHL1844 carries three point mutations in the 3´ viral RNA promoter sequence (3´-G $\overline{3}$ A,

U $\overline{5}$ C, C $\overline{8}$ U : 3´-UCAUCUUUGUCCCCAU), as originally introduced into plasmid pHL1104 (Neumann G., Hobom G, J. Gen. Virol. Jul.;76(Pt 7):1709-17 (1995)).

Plasmid construction of WSN/FPV-PB1 chimera: Original plasmids WSN-PB1 (pPolI-WSN-PB1; Neumann G. et al., Proc. Natl. Acad. Sci. USA, Aug. 3;96(16):9345-50 (1999)), and FPV-PB1 as obtained by RT-PCR from FPV vRNA using PB1 terminal primer oligonucleotides that were designed for insertion into pHH21 (Hoffmann E., Justus Liebig Universität Giessen (1997)) via *Bsm*BI cleavage (Menke A. and Hobom G., unpublished) were used as starting points. Fragments have been exchanged either through restriction cleavage at homologous unique restriction sites or following PCR reactions designed to insert a "missing" restriction site in homologous position and included within one of the primer oligonucleotides. All PCR-derived constructs have been confirmed by sequencing across the region inserted in one way or another.

Cells and viruses: Influenza viral strains A/FPV$_{Bratislava}$ (H7/N7) and A/WSN/33 (H1/N1) as well as all viral reassortants or PB1-chimeric viral constructs were grown in Madin-Darby canine kidney (MDCK) cells. Human 293T cells were used for DNA transfection and consecutive superinfection with FPV or with the various other strains used as helper virus. The resulting recombinant influenza viruses were propagated in MDCK cells. All cell lines were grown in Dulbecco´s modified Eagle's Medium (DMEM; GIBCO/BRL), supplemented with 10% fetal calf serum and antibiotics.

Generation of infectious WSN, FPV and FPV/WSN-PB1 chimeric influenza viruses: Subconfluent 293 T cells were transfected with a set of eight RNA-polymerase I plasmids yielding the eight different species of influenza vRNA molecules by *in vivo* transcription, according to Neumann et al., Proc Natl. Acad. Sci. 96, 9345-9350 (1999), together with a second set of four to five RNA-polymerase II expression plasmids for synthesis of early viral mRNA and proteins: PB1, PB2, PA, NP and (inconsistently) NS2. The latter

plasmids for expression of early FPV viral proteins (pCMV-PB1, pCMV-PB2, pCMV-PA, pCMV-NP, pCMV-NS2 (A. Menke and G. Hobom, unpublished) were used in amounts of: 1 µg/1 µg/ 0.1 µg/ 1µg/ 0.3µg, respectively, while 1 µg each was used for the set of eight RNA-polymerase I vRNA plasmids. While the entire set of FPV plasmids in this category has been obtained by RT-PCR cloning into pHH21 (Menke A. and Hobom G., unpublished), the corresponding set of WSN has been obtained from G. Neumann, Madison: pPolI-WSN-PB1, pPolI-WSN-PB2, pPolI-WSN-PA, pPolI-WSN-HA, pPolI-WSN-NP, pPolI-WSN-NA, pPolI-WSN-M, pPolI-WSN-NS. Mixtures between the two series have been used for generation of WSN-FPV viral reassortants, and for generation of WSN/FPV-PB1 chimeric viruses pPolI-WSN-PB1 has been substituted by the corresponding plasmid constructs. The combined sets of plasmids have been mixed in preparation for DNA transfection with Lipofectamin plus in a ratio of 3 µg of plasmid DNAs together with 6 µl of Lipofectamin and 8µl of Lipofectamin plus, and treated as described below. Alternatively, a set of only eight plasmids according to Hoffmann et al., Proc. Natl. Acad. Sci. 97, 6108-6113 (2000), has been used instead, again with chimeric constructs of the PB1 segment in exchange for a regular WSN-PB1 coding sequence. After 48 to 72 h the supernatant of the DNA transfected 293 T cells was used for passage on MDCK cells, and directly or thereafter also for plaque-purification and determination of the yields achieved in the generation of viral strains. Upon preparation of viral stocks the constitution in particular of PB1 vRNA as well as others was confirmed via RT-PCR analysis and diagnostic restriction cleavages of the PCR bands obtained.

<u>pHL1844 plasmid DNA transfection and influenza virus infection:</u> For pHL1844 DNA transfection we used ~3.6×10^6 subconfluent 293T cells. Briefly: 3µg of plasmid DNA in 186µl serum-free DMEM were gently mixed with 6µl Lipofectamine and 8µl of Lipofectamine Plus (GIBCO/BRL) and incubated for 30 min at room temperature. In the meantime cells were washed with serum-free medium, and the transfection mix filled up to 3

ml with medium was carefully dispersed over the cells. After 6 h of incubation the medium was changed to DMEM containing 10% FCS and further incubated for 15 h. The transfected cells were washed very carefully with PBS+ (2.5 mM $MgCl_2$; 3.4 mM $CaCl_2$ added) and superinfected with FPV helper virus at an m.o.i. of 2-3 in 1 ml of PBS+. After 1 h the cells were washed and finally resupplied with DMEM containing 10% FCS for another 8 h of incubation. A complete replication cycle of the virus takes place in this period.

Serial passage of virus containing supernatants: After 8 h of viral propagation the supernatant containing the progeny virus was collected, and after a brief centrifugation step (10 000 rpm, 5 min) for cell debris removal it was used for passaging the recombinant virus mixture onto confluent MDCK cells as described previously (Flick R. et al. RNA, Oct.;2(10):1046-57 (1996)).

CAT assay: Cell extracts of 110µl were prepared as described by Gorman *et al.* (1982). In an initial series 50µl of each cell lysate, and depending on the data obtained, also serially diluted amounts of the various cell lysates (always in parallel including material from one or more reference reactions) were mixed with 10µl of 4 mM acetyl-CoA and 8µl of fluorescent-labeled chloramphenicol (borondipyrromethane difluoride fluorophore: BODIPY CAM substrate, FLASH CAT kit, Stratagene). Samples were incubated at 37°C for 3 h. For extracting the reaction products 0.5 ml of ethyl acetate were added, and after a centrifugation step for 3 min at 13000 rpm the upper phase containing the acetylated products was transferred into new Eppendorf tubes and vacuum dried.

The resulting pellet was resuspended in 20µl ethylacetate and the reaction products were separated by thin layer chromatography (TLC plates 20/20 cm, Silica gel 60) using a solvent mixture (mobile phase) of chloroform and methanol (87:13). Finally, the reaction products were visualized by UV illumination, documented by photography and evaluated using the

WinCam system (Cybertech, Berlin). Ratios of activity have been calculated relative to reference construct pHL1844 based on three independent sets of serial dilutions of cell lysates down to yielding below 30-50% of product formation in each case. The results are summarized in Table 1 and Figures 2 and 3, right hand columns.